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Modified Alizarin Red S-Alcian Blue Staining for Reptilian Skeleton

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Abstract

Skeletal staining is an important method in anatomical study. The aim of the research was to develop staining and clearing method of Reptilian skeleton using Alizarin Red S-Alcian Blue. The specimen were eviscerated, fixed, stained, cleared, and keep in glycerine solution. This modified double-staining has successfully stain bone and cartilage of Reptilian.

Keywords: Skeleton, Alizarin Red S, Alcian Blue, Reptilia, Double-staining

Introduction

Skeleton are supporting system for vertebrate body. The skeleton support body, provides for attachment muscles, houses for brain and nerve, and serve for locomotion (Kardong, 2002). Staining of animal skeleton is important in anatomical study. A lot of papers report methods for skeleton staining. Clearing and staining of skeletal system are aimed for demonstrate of animal bone and cartilage. This is a routine method in teratological study and has been widely used for study in fetal and young animal, especially rodent (Erdogan *et al*., 1995).

Alizarin Red S-Alcian Blue is one of the most successful double staining methods for fetal and young rodent skeleton. Application of this method in mature reptile, however, is still scarce (Saralamoli *et al*., 2015). This paper will explain modified Alizarin Red S-Alcian Blue for successful staining in mature reptilian skeleton.

Materials and Methods

Animals used in this study are *Ahaetulla prasina*, *Gecko gecko*, *Trachemys scripta elegans,* and *Draco volans.* Equipments used in this study are the surgical scissors, scalpels, and a glass container (jar). Materials used in this study are Alizarin Red S-Alcian Blue, ethanol, distilled water, glacial acetic acid, glycerin and KOH.

The method described in this paper is a modification from Inouye (1976) that has been used on small vertebrates. Inouye was the first researcher who successfully use staining methods for bone and cartilage at once with satisfactory result. Inouye (1976) use a double staining method for bone and cartilage in mice for teratological study. Using this method cartilage stained blue, bone stained red, whereas muscles and other tissues are transparent. Appication of this method for mature and larger vertebrate required a modification of flaking skin and muscles into thinner fragments, aditional and prolonged fixation, fat removal, prolonged staining, and purification. These are because adult tissue and larger vertebrates have massive muscle, thicker skin and dense connective tissue, thus staining and clearing process are more difficult.

Staining and clearing reptilian specimens includes several stages. The first step is exfoliation. After the scales removed, the specimens were fixed in glass tubes containing 95% alcohol for 3 days. After the skin and muscle in the specimen removed by scissors and a scalpel, the specimen immersed again in the new 95% ethanol for 3 days. To remove fat, specimens are put in tubes containing acetone for 4 days with new acetone replacement on the second day. After that the specimens were kept for 5 days in a solution of the dye at a temperature of 37 ° C (1 volume of 0.3% Alcian Blue in 70% ethanol + 1 volume of 0.1% Alizarin Red S in 95% ethanol + 1 volume of glacial acetic acid + 17 volume 70% ethanol). The specimen is washed in water and then soaked in a solution of 1% KOH for 5 days (until transparent) and then shaked on a shaker in a mixture of glycerin and KOH 1% with a ratio of 20%: 80%; 50%: 50%; 80%: 20%. Then the specimens are preserved in pure glycerine.

Result and Discussion

This research has succeeded in developing a method for staining and clearing reptile skeleton specimen skeleton (bone and cartilage) with Alizarin Red S - Alcian Blue (Figure 1, Figure 2, Figure 3, Figure 4 and Figure 5). Figure 1 and Figure 2 shows the skeleton of a snake *Ahaetulla prasina.* The preparation showed the bones of the skull and vertebrae. The snake specimen shows a special adaptation of vertebrate skeleton, the vertebrae are huge numbers (± 300 vertebra vertebrae) suitable for terrestrial life without limbs. Figure 3 shows tail skeleton of *Gecko gecko*. The preparation shows the

structure of the tail vertebrae in autotomous animals that can spontaneously casting off their tail. Figure 4 shows the tail skeleton of turtles (*Trachemys scripta elegans*). Figure 5 shows the tail skeleton of Draco volans. The turtle and *Draco volans* shows the structure of non-autotomous tail vertebrae.

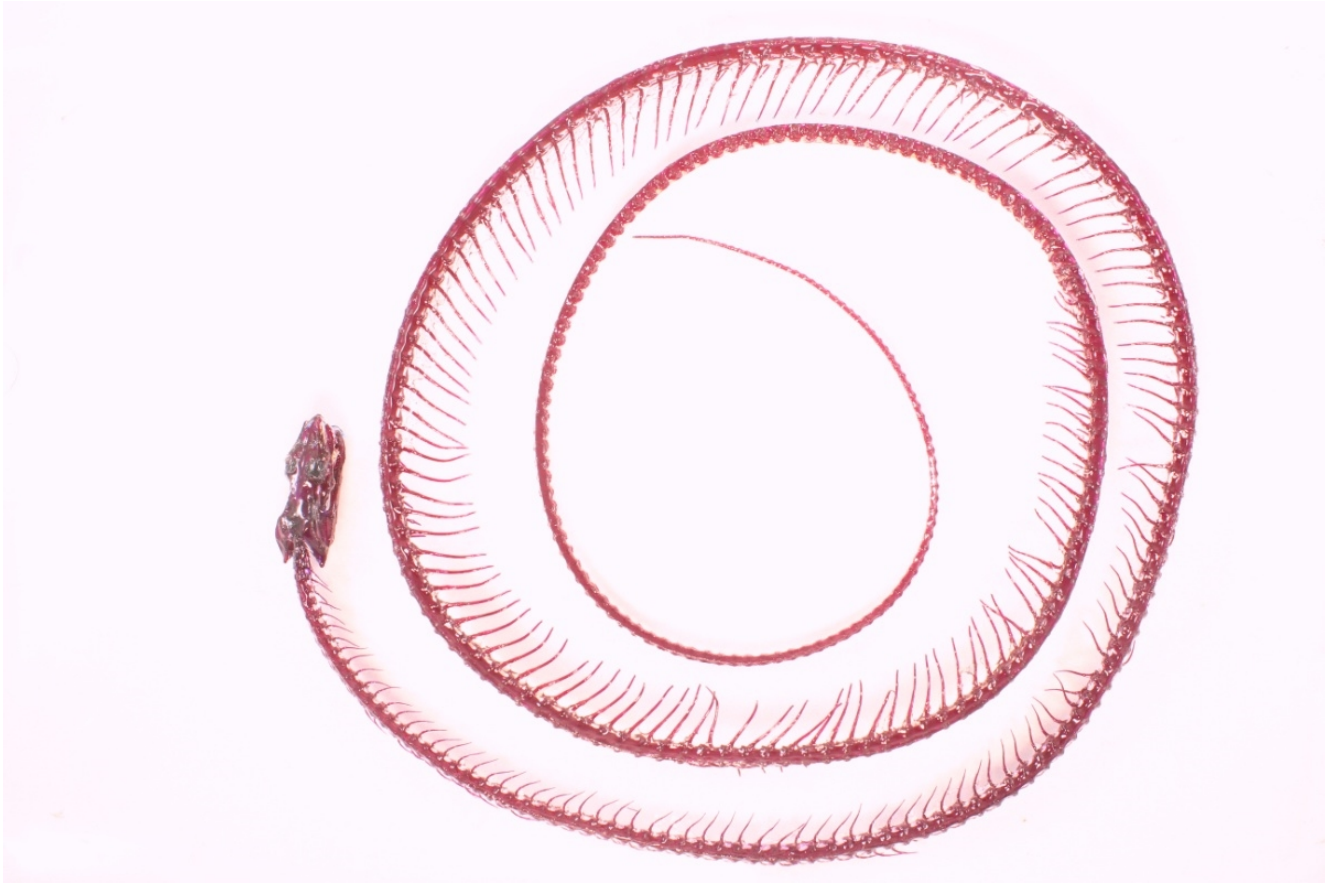

Figure 1. Structure of snake *Ahaetulla prasina*. Note skull and spine with ribs.

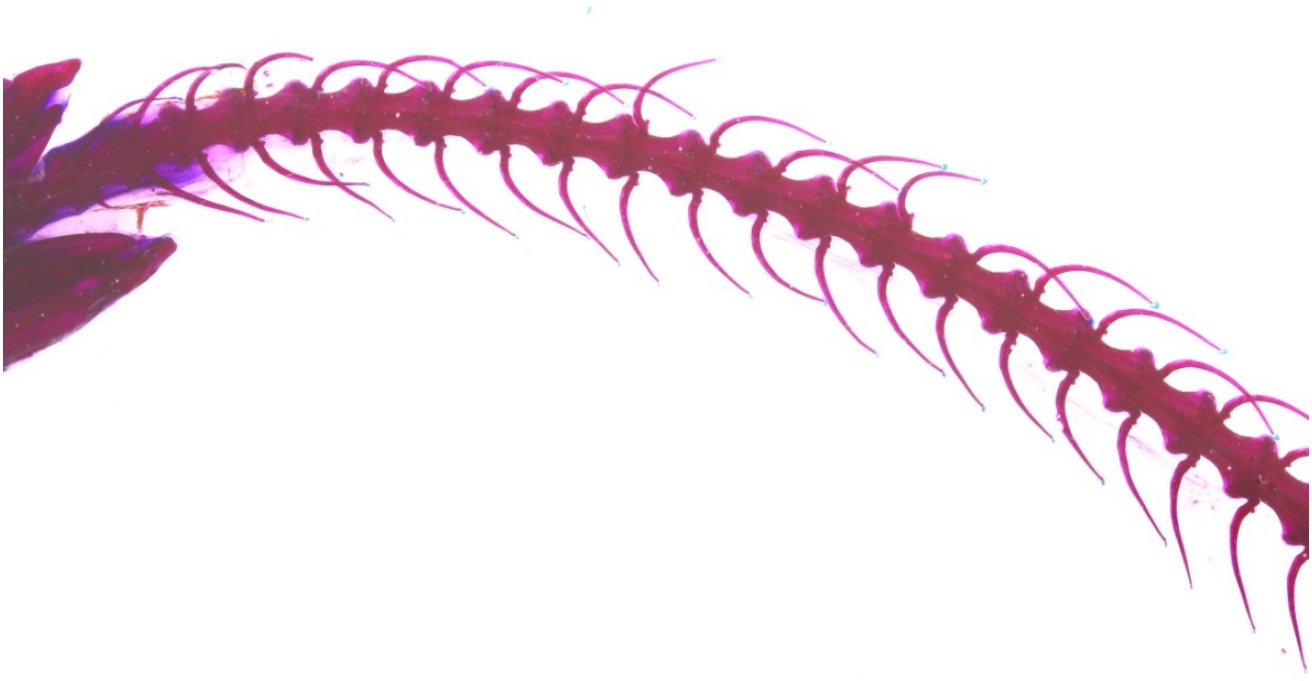

Figure 2. High magnification of skeletal structure of snake *Ahaetulla prasina.*

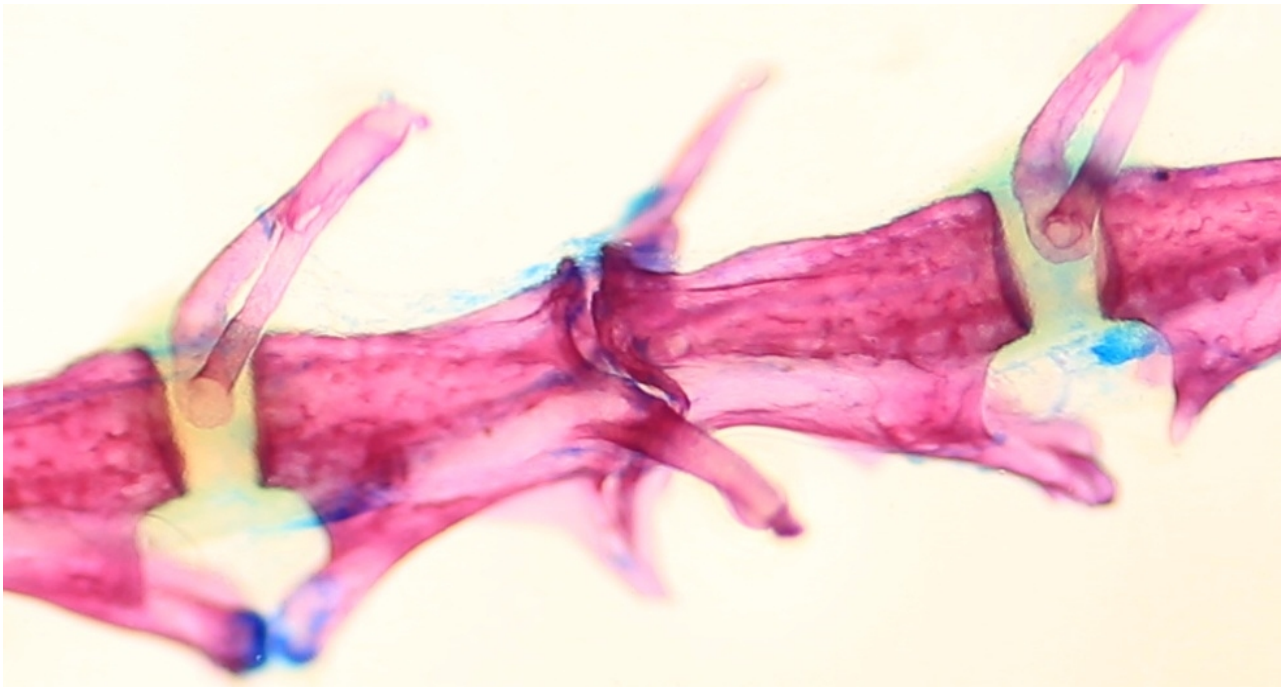

Figure 3. High magnification of tail skeleton of *Gecko gecko*. Note the presence of autotomous split in the plain where the tail broke in autotomy.

Figure 4. High magnification of tail skeleton of turtles. Turtles cannot autotomized their tail therefore no autotomous split in this vertebrae.

Figure 5. High magnification of tail skeleton of *Draco volans.*

The results show that all specimen are clearly visible. Bones stained red whereas cartilage stained blue. Muscle and connective tissue are transparent/clear by a clearing process with KOH. From the specimens we can observe the vertebrae and its processus, intervertebral discs, ribs, autotomus split, and other details.

In this method, the most important aspect for successful staining is exfoliation of skin and muscle, as well as fixation stages. Remove the skin and muscle as much as possible. But it should be noted that exfoliation skin and muscles are not cut the skeleton itself. Vertebrae have many processes. It is often that the disposal of the skin and muscle also cutting processus. Fixation and the removal of fat must be prolonged compared to Inouye method for fixative solution penetrate the tick tissue. Different specimen takes different times for fixation period, the removal of fat, staining and clearing. It depends on the species, maturity (embryonic or adult), body size, and organ/body part made preparation. The more ticker and mature the tissue, the more times are needed. In general, larger animals and more mature require fixation longer period, longer removal of fat, longer staining and and longer clearing. Duration time of fixation, removal of fat, staining and clearing in this study can be used as a guidance in application of this method to other animals.

Whole body staining and clearing method have certain advantages over methods in which the carcass is macerated and the bones are separated and dried. Among these advantages are: (1) there is no chance of losing the small bones, (2) all bones are retained in their original position, (3) there is no chance of wrongly identifying similar bones, (4) in the finished preparations the bones, after identification, may be disarticulated and examined from all angles, equally as well as in dried preparations, and (5) many animals may be processed together without danger of mixing their bones, a great saving in time and effort (Green, 1952).

Conclusion

This research has successfully modified Alizarin Red S-Alcian Blue to applied on larger and mature animal (Reptilia) with satisfied result.

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